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IN THE UNITED STATES PATENT AND TRADEMARK OFFICE

In re **PROVISIONAL** Patent Application of: Mark T. Gladwin et al.

Title: TREATMENT OF CARDIOVASCULAR CONDITIONS WITH NITRITE

Docket No.: 1662.026PV2

MAIL STOP PROVISIONAL APPLICATION
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P.O. Box 1450
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X A PROVISIONAL Patent Application comprising:
X Specification (34 pgs, including formal claim 1 and Statements of the Invention numbered 1 through 22 and a 1 page Abstract)
X 7 Sheet(s) of drawing(s).
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_ A check in the amount of $160.00 to cover the Provisional Filing Fee.
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PROVISIONAL APPLICATION COVER SHEET

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Docket Number	1662.026PV2	Type a plus sign (+) inside this box >	+
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INVENTOR(s)/APPLICANT(s)

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TITLE OF THE INVENTION (280 characters max)

TREATMENT OF CARDIOVASCULAR CONDITIONS WITH NITRITE

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STATE	Minnesota	ZIP CODE	55402	COUNTRY	United States of America

ENCLOSED APPLICATION PARTS (check all that apply)

XXX	Specification	Number of Pages	34		Small Entity Statement	
XXX	Drawing(s)	Number of Sheets	7		Other (specify)	Declaration and Power of Attorney (4 pgs.) and Assignment (2 pgs.) and Recordation Form Cover Sheet (1 pg) and Communication (1 pg.)

METHOD OF PAYMENT (check one)

	A check or money order is enclosed to cover the Provisional filing fees	PROVISIONAL FILING FEE AMOUNT	$160.00
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The invention was made by an agency of the United States Government or under a contract with an agency of the United States Government.

No.

XXX Yes, the name of the U.S. Government agency and the Government contract number are: NIH and Clinical Center and NHLBI intramural NIH funds.

Respectfully submitted,

SIGNATURE _____ Date October 14, 2003

TYPED OR PRINTED NAME Peter L. Malen REGISTRATION NO. 44,894

Additional inventors are being named on separately numbered sheets attached hereto.

PROVISIONAL APPLICATION FILING ONLY

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PATENT

IN THE UNITED STATES PATENT AND TRADEMARK OFFICE

Applicant:	Mark T. Gladwin et al.	Examiner: Unknown
Serial No.:	Herewith	Group Art Unit: Unknown
Filed:	October 14, 2003	Docket: 1662.026PV2
Title:	TREATMENT OF CARDIOVASCULAR CONDITIONS WITH NITRITE	

COMMUNICATION

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P.O. Box 1450
Alexandria, VA 22313-1450

Applicants are submitting herewith a Declaration and Power of Attorney and an Assignment document to be filed with this provisional application. Although this is a provisional application, the executed Declaration and Power of Attorney and the Assignment documents are submitted as if the application were a utility application.

The Commissioner is hereby authorized to charge any additional required fees to Deposit Account No. 19-0743, if deemed necessary.

The Examiner is invited to contact the Applicant's Representative at the below-listed telephone number if there are any questions regarding this communication.

Respectfully submitted,

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SLWK No: 1662.026PV2

UNITED STATES PROVISIONAL PATENT APPLICATION

TREATMENT OF CARDIOVASCULAR CONDITIONS WITH NITRITE

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TREATMENT OF CARDIOVASCULAR CONDITIONS WITH NITRITE

Statement of Government Rights

The invention was made with the support of a grant from the Government of the United States of America (NIH grant HL58091 and Clinical Center and NHLBI intramural NIH funds). The Government may have rights in the invention.

Background of the Invention

The last decade has seen an increase in the understanding of the critical role nitric oxide (NO) plays in vascular homeostasis. The balance between production of NO and scavenging of NO determines NO bioavailability, and this balance is carefully maintained in normal physiology. However, when this balance is disrupted, for example by NO scavenging by cell-free hemoglobin (Reiter et al., 2002) a variety of complications can occur. A current focus of research attempts to treat the vascular complications common to many chronic hemolytic conditions, such as pulmonary hypertension, cutaneous ulceration and acute and chronic renal failure. Similarly, a number of clinical diseases and therapies such as acute hemolytic crises, hemolysis during cardiopulmonary bypass procedures, transfusion of aged blood, and myoglobinuria following muscle infarction are often complicated by acute pulmonary and systemic hypertension, acute renal failure, intravascular thrombosis, ischemic central nervous system events and/or death. To date, effective treatments for these conditions are lacking.

In vivo plasma levels of nitrite have been reported to range from 150 to 1000 nM, and the nitrite concentration in aortic ring tissue has been reported to be in excess of 10 μM. (Rodriguez et al., 2003; Gladwin et al., 2000; and Rassaf et al., 2003) This potential storage pool for NO is in excess of plasma S-nitrosothiols, which has been reported to be less than 10 nM in human plasma. (Rassaf et al., 2003; Rassaf et al., 2002a; Rassaf et al., 2002b; and Schechter et al., 2002) Mechanisms have been proposed for the *in vivo* conversion of nitrite to NO, for example, by enzymatic reduction by xanthine oxidoreductase or by non-enzymatic

disproportionation/acidic reduction. (Millar et al., 1997; Millar et al., 1998; Godber et al., 2000; Zhang et al., 1998; Li et al., 2001; Li et al., 2003; Zweier et al., 1995; Zweier et al., 1999; and Samouilov et al., 1998) At high concentrations, nitrite has been reported to be a vasodilator *in vitro*. (Ignarro et al., 1980; Ignarro, 1981; Moulds et al., 1981; Gruetter et al, 1981; Matsunaga et al., 1989; and Laustiola et al., 1991) Arterial-to-venous gradients of nitrite across the human forearm at rest and during regional NO synthase inhibition have been observed, with increased consumption of nitrite occurring with exercise. (Gladwin et al., 2000; and Cicinelli et al., 1999) Kelm and colleagues have reported that large artery-to-vein gradients of nitrite form across the human forearm during NO synthase inhibition. (Lauer et al., 2001) Unlike the more simple case of oxygen extraction across a vascular bed, nitrite may be both consumed, as evidenced by artery-to-vein gradients during NO synthase inhibition and exercise, and produced in the vascular bed by endothelial nitric oxide synthase-derived NO reactions with oxygen.

However, when Lauer and colleagues infused nitrite into the forearm circulation of human subjects, they reported no vasodilatory effects. (Lauer et al., 2001) Lauer et al. reported that a "complete lack of vasodilator activity of intraartierial infusions of nitrite clearly rules out any role for this metabolite in NO delivery" and concluded that "physiological levels of nitrite are vasodilator-inactive." Furthermore, Rassaf and colleagues also failed to find a vasodilatory effect in humans following infusion of nitrite. (Rassaf et al., 2002b) Thus, *in vivo* studies have concluded that physiological levels of nitrites do not serve as a source for NO, and that physiological levels of nitrites do not have a role in regulating blood pressure.

Therefore, there is a still need for methods to modulate the NO system and to regulate blood pressure and improve blood flow in people needing such treatment, for example, people with hemolytic conditions such as sickle cell anemia.

Summary of the Invention

These and other needs are met by the current invention. It has been surprisingly discovered that administration of nitrite to patients causes a reduction in blood pressure and an increase in blood flow to tissues, for example, to tissues in regions of low oxygen tension.

Accordingly, the present invention provides a method for decreasing a patient's blood pressure, including administering to the patient sodium nitrite at about 36 μmoles per minute into the forearm brachial artery.

The present invention additionally provides a method for increasing blood flow to a tissue of a patient, including administering to the patient an effective amount of pharmaceutically-acceptable nitrite so as to increase blood flow to a tissue of the patient. The blood flow may be specifically increased in tissues in regions of low oxygen tension.

The present invention also provides a method for decreasing a patient's blood pressure, comprising administering to the patient an effective amount of pharmaceutically-acceptable nitrite so as to decrease the patient's blood pressure.

The present invention further provides a method for treating a patient having a condition associated with elevated blood pressure, including administering to the patient an effective amount of pharmaceutically-acceptable nitrite so as to treat at least one vascular complication associated with the elevated blood pressure.

Also provided is a method for treating a patient having a hemolytic condition, including administering to the patient an effective amount of pharmaceutically-acceptable nitrite so as to treat at least one vascular complication associated with the hemolytic condition.

The invention further provides a method for treating a patient having a condition associated with elevated blood pressure in the lungs, *e.g.* pulmonary hypertension, including administering to the patient an effective amount of pharmaceutically-acceptable nitrite. In some embodiments, this includes treating a patient having neonatal pulmonary hypertension. In some embodiments, this includes treating a patient having primary and/or secondary pulmonary

hypertension. In some embodiments for treating patients having a condition associated with elevated blood pressure in the lungs, the nitrite is nebulized.

Brief Description of the Figures

Figure 1. Figure 1 depicts hemodynamic and metabolic measurements at baseline and during exercise in 18 subjects. **Figure 1A** depicts effects without inhibition of NO synthesis. **Figure 1B** depicts effects with inhibition of NO synthesis.

Figure 2. Figure 2 depicts effects of infusion of sodium nitrite in bicarbonate-buffered normal saline into the brachial arteries of 18 healthy subjects. **Figure 2A** depicts effects without inhibition of NO synthesis. **Figure 2B** depicts effects with inhibition of NO synthesis.

Figure 3. Figure 3 depicts effects of infusion of low-dose sodium nitrite into the brachial arteries of 10 healthy subjects at baseline and during exercise, without and with inhibition of NO synthesis. **Figure 3A** depicts forearm blood flow at baseline and following a five-minute infusion of $NaNO_2$. **Figure 3B** depicts forearm blood flow with and without low-dose nitrite infusion at baseline and during L-NMMA infusion with and without exercise stress. **Figure 3C** depicts venous levels of nitrite from the forearm circulation at the time of blood flow measurements. **Figure 3D** depicts venous levels of S-nitroso-hemoglobin and iron-nitrosyl-hemoglobin at baseline and following nitrite infusion during exercise stress.

Figure 4A depicts formation of iron-nitrosyl-hemoglobin and S-nitroso-hemoglobin. **Figure 4B** depicts a comparison between formation of NO-hemoglobin adducts with hemoglobin-oxygen saturation in the human circulation during nitrite infusion.

Figure 5A depicts NO release following nitrite injections into solutions of PBS, deoxygenated red blood cells, and oxygenated red blood cells. **Figure 5B** depicts the rate of NO formation from nitrite mixed with PBS and oxygenated and deoxygenated red blood cells.

Detailed Description of the Invention

It has been surprisingly discovered that administration of pharmaceutically-acceptable salts of nitrite are useful in the regulation of the cardiovascular system. It has also been surprisingly discovered that nitrite is reduced to nitric oxide *in vivo*, and that the nitric oxide produced thereby is an effective vasodilator. These discoveries provide useful methods to prevent and treat conditions associated with the cardiovascular system, for example, high blood pressure. These discoveries also provide useful methods to increase blood flow to tissues, for example, to tissues in regions of low oxygen tension.

Accordingly, the present invention provides a method for decreasing a patient's blood pressure, including administering to the patient sodium nitrite at about 36 μmoles per minute into the forearm brachial artery.

The present invention also provides a method for decreasing a patient's blood pressure, including administering to the patient an effective amount of pharmaceutically-acceptable nitrite so as to decrease the patient's blood pressure.

The present invention further provides a method for treating a patient having a condition associated with elevated blood pressure, including administering to the patient an effective amount of pharmaceutically-acceptable nitrite so as to treat at least one vascular complication associated with the elevated blood pressure.

Also provided is a method for treating a patient having a hemolytic condition, including administering to the patient an effective amount of pharmaceutically-acceptable nitrite so as to treat at least one vascular complication associated with the hemolytic condition.

The present invention additionally provides a method for increasing blood flow to a tissue of a patient, including administering to the patient an effective amount of pharmaceutically-acceptable nitrite so as to increase blood flow to a tissue of the patient.

The present invention also provides a method for producing an amount of NO in a patient effective the decrease the patient's blood pressure, including administering a pharmaceutically-acceptable nitrite to the patient.

The present invention further provides a pharmaceutical composition comprising an effective amount of a pharmaceutically-acceptable nitrite and a carrier.

In some embodiments of the invention, the vascular complication is one or more selected from the group consisting of pulmonary hypertension (including neonatal pulmonary hypertension, primary pulmonary hypertension, and secondary pulmonary hypertension), systemic hypertension, cutaneous ulceration, acute renal failure, chronic renal failure, intravascular thrombosis, an ischemic central nervous system event, and death.

In some embodiments of the invention, nitrite is administered to neonates to treat pulmonary hypertension.

In some embodiments of the invention, the hemolytic condition is one or more selected from the group consisting of sickle cell anemia, thalassemia, hemoglobin C disease, hemoglobin SC disease, sickle thalassemia, hereditary spherocytosis, hereditary elliptocytosis, hereditary ovalcytosis, glucose-6-phosphate deficiency and other red blood cell enzyme deficiencies, paroxysmal nocturnal hemoglobinuria (PNH), paroxysmal cold hemoglobinuria (PCH), thrombotic thrombocytopenic purpura/hemolytic uremic syndrome (TTP/HUS), idiopathic autoimmune hemolytic anemia, drug-induced immune hemolytic anemia, secondary immune hemolytic anemia, non-immune hemolytic anemia caused by chemical or physical agents, malaria, falciparum malaria, bartonellosis, babesiosis, clostridial infection, severe haemophilus influenzae type b infection, extensive burns, transfusion reaction, ryabdomyolysis (myoglobinemia), transfusion of aged blood, cardiopulomonary bypass, and hemodialysis.

In some embodiments of the invention, the decreased blood flow to the tissue is caused directly or indirectly by at least one condition selected from the group consisting of: sickle cell anemia, thalassemia, hemoglobin C disease, hemoglobin SC disease, sickle thalassemia, hereditary spherocytosis, hereditary elliptocytosis, hereditary ovalcytosis, glucose-6-phosphate deficiency and other red blood cell enzyme deficiencies, paroxysmal nocturnal hemoglobinuria (PNH), paroxysmal cold hemoglobinuria (PCH), thrombotic thrombocytopenic

purpura/hemolytic uremic syndrome (TTP/HUS), idiopathic autoimmune hemolytic anemia, drug-induced immune hemolytic anemia, secondary immune hemolytic anemia, non-immune hemolytic anemia caused by chemical or physical agents, malaria, falciparum malaria, bartonellosis, babesiosis, clostridial infection, severe haemophilus influenzae type b infection, extensive burns, transfusion reaction, ryabdomyolysis (myoglobinemia), transfusion of aged blood, transfusion of hemoglobin, transfusion of red blood cells, cardiopulmonary bypass, coronary disease, cardiac ischemia syndrome, angina, iatrogenic hemolysis, angioplasty, myocardial ischemia, tissue ischemia, hemolysis caused by intravascular devices, hemodialysis, pulmonary hypertension, systemic hypertension, cutaneous ulceration, acute renal failure, chronic renal failure, intravascular thrombosis, and an ischemic central nervous system event.

In some embodiments of the invention, the tissue is an ischemic tissue. In some embodiments of the invention, the administration is parenteral, oral, bucal, rectal, *ex vivo*, or intraocular. In some embodiments of the invention, the administration is peritoneal, intravenous, intraarterial, subcutaneous, inhaled, or intramuscular. In some embodiments of the invention, the nitrite is administered to the patient in an environment of low oxygen tension. In some embodiments of the invention, the nitrite is administered as a pharmaceutically-acceptable salt of nitrite. In some embodiments of the invention, the nitrite is administered as sodium nitrite. In some embodiments of the invention, the nitrite is administered in combination with at least one additional active agent. In some embodiments of the invention, the patient is a mammal. In some embodiments of the invention, the patient is a human.

The invention further provides a method for treating a patient having a condition associated with elevated blood pressure in the lungs, *e.g.* pulmonary hypertension, including administering to the patient an effective amount of pharmaceutically-acceptable nitrite. In some embodiments, this includes treating a patient having neonatal pulmonary hypertension. In some embodiments, this includes treating a patient having primary and/or secondary pulmonary hypertension. In some embodiments for treating patients having a condition associated with elevated blood pressure in the lungs, the nitrite is nebulized.

The terms used herein should be accorded their standard definitions. For example, one of skill in the art can obtain definitions for the terms used herein in dictionaries and reference textbooks, for example, Stedman's Medical Dictionary (26th Edition, Williams and Wilkins, Editor M. Spraycar, 1995), The New Oxford American Dictionary (Oxford University Press, Eds E. Jewell and F. Abate, 2001), Molecular Cloning: A Laboratory Manual (Sambrook et al., 3rd Ed., Cold Spring Harbor Laboratory Press, (2001), and Hawley's Condensed Chemical Dictionary, 11th Ed., Eds. N. I. Sax and R. J. Lewis, Sr., Van Nostrand Reinhold, New York, New York, 1987).

Methods of Use

Nitrites can be administered to a patient to increase blood flow to a tissue of a patient, for example, to increase blood flow to a tissue with low oxygen tension; to cause vasodilation; to decrease a patient's blood pressure; to treat a patient having a condition associated with elevated blood pressure; to treat a hemolytic condition; and/or to treat vascular complications associated with treatments or conditions that cause hemolysis.

The vasodilator properties of nitrite and the mechanisms for its bioactivation were investigated as described herein. Sodium nitrite was infused at 36 μmoles per minute into the forearm brachial artery of 18 normal volunteers, resulting in a regional nitrite concentration of 222 μM and, surprisingly, an immediate 175% increase in resting forearm blood flow. Increased blood flow was observed at rest, during NO synthase inhibition and with exercise. The nitrite infusion also surprisingly resulted in increased tissue perfusion, as demonstrated by increases in venous hemoglobin-oxygen saturation, partial pressure of oxygen, and pH. Increased systemic concentrations of nitrite (16 μM) significantly reduced mean arterial blood pressure.

In an additional ten subjects, the dose of nitrite was reduced 2-logs, resulting in a forearm nitrite concentration of 2 μM at rest and 1 μM during exercise (Figure 3). These concentrations of nitrite surprisingly significantly increased blood flow at rest and during NO synthase inhibition, with and without exercise.

Nitrite infusions were associated with the rapid formation of erythrocyte iron-nitrosyl-hemoglobin, and to a lesser extent, S-nitroso-hemoglobin across the forearm vasculature. Formation of these NO-Hb adducts was inversely proportional to the oxyhemoglobin saturation. Additionally, vasodilation of rat aortic rings and the formation of both NO gas and NO-modified hemoglobin from the nitrite reductase activity of deoxyhemoglobin and deoxygenated erythrocytes was observed, a result that links tissue hypoxia, hemoglobin allostery, and nitrite bioactivation. These results indicate that physiological levels of blood and tissue nitrite are a major bioavailable pool of NO that contributes to vaso-regulation and provide a mechanism for hypoxic vasodilation via reaction of vascular nitrite with deoxygenated heme proteins in tissue and/or the erythrocyte.

The findings described herein that administration of nitrite reduces blood pressure and increases blood flow are unexpected and surprising because published reports to date teach the art worker that physiological levels of nitrites, when administered to patients, lack vasodilatory properties.

A mechanism of iron-nitrosyl- and S-nitroso-hemoglobin formation *in vivo*

The levels of both iron-nitrosyl- and S-nitroso-hemoglobin formed *in vivo* in this study are striking. During a transit time of less than 10 seconds through the forearm circulation during exercise, infused nitrite (200 μM regional concentration) produced approximately 750 nM iron-nitrosyl-hemoglobin and 200 nM SNO-Hb. The formation of both NO-hemoglobin adducts was inversely correlated with hemoglobin-oxygen saturation, which fell during exercise stress, measured from the antecubital vein by co-oximetry (for iron-nitrosyl-hemoglobin $r=-0.7$, $P<0.0001$; for S-nitroso-hemoglobin $r=-0.45$, $P=0.04$; Figure 4B). Addition of 200 μM nitrite to whole blood at different oxygen tensions (0-100%) recapitulated the *in vivo* data with increasing concentrations of iron-nitrosyl hemoglobin being formed at lower oxygen tensions (for iron-nitrosyl-hemoglobin $r=-0.968$, $P<0.0001$; for S-nitroso-hemoglobin $r=-0.45$, $P=0.07$; data not shown), strongly suggesting that the NO and SNO formation was dependent on the reaction of nitrite with deoxyhemoglobin.

These data are consistent with the reaction of nitrite with deoxyhemoglobin to form NO and iron-nitrosyl-hemoglobin. (Doyle et al., 1981) Nitrite is first reduced to form NO and methemoglobin with a rate constant of 2.9 $M^{-1}sec^{-1}$ (measured at 25°C, pH 7.0). This reaction will be pseudo-first order, governed by the amounts (20 mM) of intra-erythrocytic hemoglobin, and limited by the rate of nitrite uptake by the erythrocyte membrane. NO then binds to deoxyhemoglobin to form iron-nitrosyl-hemoglobin, escapes the erythrocyte, or reacts with other higher oxides, such as NO_2, to form N_2O_3 and S-nitroso-hemoglobin.

The formation of significant amounts of S-nitroso-hemoglobin *in vivo* during nitrite infusion were also observed. Luschinger and colleagues (Luschinger et al., 2003) recently proposed that nitrite reacts with deoxyhemoglobin to make iron-nitrosyl-hemoglobin, with subsequent "transfer" of the NO to the cysteine 93 to form S-nitroso-hemoglobin mediated by reoxygenation and quaternary T to R transition of hemoglobin. However, a direct transfer of NO from the heme to the thiol requires NO oxidation to NO+ and such "cycling" has not been reproduced by other research groups. Fernandez and colleagues have recently suggested that nitrite catalyzes the reductive nitrosylation of methemoglobin by NO, a process that generates the intermediate nitrosating species dinitrogen teraoxide (N_2O_3). (Fernandez et al., 2003) However, nitrite reactions with hemoglobin provide ideal conditions for NO and S-nitrosothiol generation along the oxygen gradient as nitrite reacts with deoxyhemoglobin to form NO and with oxyhemoglobin to form nitrogen dioxide (NO_2) radical. NO_2 participates in radical-radical reactions ($k=10^9$ $M^{-1}sec^{-1}$) with NO to form N_2O_3 and S-nitrosothiol. Additional chemistry of nitrite with hemoglobin produces reactive oxygen metabolites (ie. superoxide and hydrogen peroxide; Watanabe et al., 1981; Kosaka et al., 1982; and Kosaka et al., 1987) Chemistry involving such NO radical- oxygen radical reactions provides competitive pathways for S-nitrosothiol formation in the presence of high affinity NO sinks, such as hemoglobin.

Physiological considerations

The last decade has seen an increase in the understanding of the critical role nitric oxide (NO) plays in vascular homeostasis. The balance between production of NO and scavenging of NO determines NO bioavailability, and this balance is carefully maintained in normal physiology. The homeostatic, vasoregulatory system is apparently fine-tuned to scavenge excess NO to limit gross endocrine actions while allowing for sufficient local NO necessary for regional tonic vasodilation. However, rapid NO scavenging by cell-free hemoglobin disrupts this balance. (Reiter et al., 2002) Under normal physiological conditions, hemoglobin is rapidly and effectively cleared by the hemoglobin scavenger system. However, chronic hemolytic conditions, such as sickle cell disease, result in the daily release of substantial quantities of hemoglobin into the vasculature, suggesting that cell-free hemoglobin may have major systemic effects on NO bioavailability. A current focus of research attempts to explain and treat the vascular complications common to many chronic hemolytic conditions, such as pulmonary hypertension, cutaneous ulceration and acute and chronic renal failure. Similarly, a number of clinical diseases and therapies such as acute hemolytic crises, hemolysis during cardiopulmonary bypass procedures, transfusion of aged blood, and myoglobinuria following muscle infarction are often complicated by acute pulmonary and systemic hypertension, acute renal failure, intravascular thrombosis, ischemic central nervous system events and/or death.

It is demonstrated herein that nitrite produces vasodilation in humans associated with nitrite reduction to NO by deoxyhemoglobin. Remarkably, systemic levels of 16 μM resulted in systemic vasodilation and decreased blood pressure, and regional forearm levels of only 1-2 μM significantly increased blood flow at rest and with exercise stress. Furthermore, conversion of nitrite to NO and S-nitrosothiol was mediated by reaction with deoxyhemoglobin, providing a mechanism for hypoxia-regulated catalytic NO production by the erythrocyte or endothelial/tissue heme proteins. While high concentrations of hemoglobin in red cells, coupled with the near diffusion-limited reaction rates ($\sim 10^7\ M^{-1}s^{-1}$) of NO with hemoglobin, seem to prohibit NO from being exported from the red blood cell, the

data presented herein argue to the contrary. While not intending to be limiting, perhaps the unique characteristics of the erythrocyte membrane, with a submembrane protein and methemoglobin-rich microenvironment, and the relative lipophilic nature of NO, allow compartmentalized NO production at the red blood cell membrane. This, coupled with the small yields of NO necessary for vasodilation, could account for the export of NO despite these kinetic constraints.

Three factors uniquely position nitrite, rather than S-nitrosothiol, as the major vascular storage pool of NO: 1) nitrite is present in substantial concentrations in plasma, erythrocytes and in tissues; 2) nitrite is relatively stable, because it is not readily reduced by intracellular reductants (as are S-nitrosothiols) and its reaction rate with heme proteins is 10,000 times less than that of authentic NO; and 3) nitrite is only converted to NO by reaction with deoxyhemoglobin (or presumably deoxy-myoglobin, -cytoglobin, -neuroglobin and presumably other oxygen binding heme proteins) and its "leaving group" is the met(ferric)heme protein which will limit scavenging and inactivation of NO. Therefore, this pool provides the ideal substrate for NO generation along the physiological oxygen gradient, providing a novel mechanism for hypoxic vasodilation.

Therapeutic application of nitrite will provide selective vasodilation to hypoxemic and ischemic tissue and will be useful to treat hemolytic conditions such as sickle cell disease, where free hemoglobin released during hemolysis scavenges NO and disrupts NO-dependent vascular function. Nitrite will not only inhibit the ability of free hemoglobin to scavenge NO by oxidizing it to methemoglobin but will also generate NO in tissue beds with low oxygen tension.

Formulations and Administration

Nitrites, including their salts, are administered to a patient. Administration of the nitrites in accordance with the present invention may be in a single dose, in multiple doses, and/or in a continuous or intermittent manner, depending, for example, upon the recipient's physiological condition, whether the purpose of the administration is therapeutic or prophylactic, and other factors known to skilled practitioners. The administration of the nitrites may be essentially continuous over

a preselected period of time or may be in a series of spaced doses. The amount administered will vary depending on various factors including, but not limited to, the condition to be treated and the weight, physical condition, health, and age of the patient. Such factors can be determined by a clinician employing animal models or other test systems that are available in the art.

To prepare the nitrites, nitrites are synthesized or otherwise obtained and purified as necessary or desired. In some embodiments of the invention, the nitrite is a pharmaceutically-acceptable salt of nitrite, for example, sodium nitrite. In some embodiments of the invention, the nitrite is not ethyl nitrite. In some embodiments of the invention, the sodium nitrite is not on a medical devise, for example, not on a stent. In some embodiments of the invention, the nitrite is not in the form of a gel. The nitrites can be adjusted to the appropriate concentration, and optionally combined with other agents. The absolute weight of a given nitrite included in a unit dose can vary. In some embodiments of the invention, the nitrite is administered as a salt of an anionic nitrite with a cation, for example, sodium, potassium, or arginine.

One or more suitable unit dosage forms including the nitrite can be administered by a variety of routes including topical, oral, parenteral (including subcutaneous, intravenous, intramuscular and intraperitoneal), rectal, dermal, transdermal, intrathoracic, intrapulmonary and intranasal (respiratory) routes.

The formulations may, where appropriate, be conveniently presented in discrete unit dosage forms and may be prepared by any of the methods known to the pharmaceutical arts. Such methods include the step of mixing the nitrite with liquid carriers, solid matrices, semi-solid carriers, finely divided solid carriers or combinations thereof, and then, if necessary, introducing or shaping the product into the desired delivery system. By "pharmaceutically acceptable" it is meant a carrier, diluent, excipient, and/or salt that is compatible with the other ingredients of the formulation, and not deleterious or unsuitably harmful to the recipient thereof. The therapeutic compounds may also be formulated for sustained release, for example, using microencapsulation (see WO 94/ 07529, and U.S. Patent No.4,962,091).

The nitrites may be formulated for parenteral administration (*e.g.*, by injection, for example, bolus injection or continuous infusion) and may be presented in unit dose form in ampoules, pre-filled syringes, small volume infusion containers or in multi-dose containers. Preservatives can be added to help maintain the shelve life of the dosage form. The nitrites and other ingredients may form suspensions, solutions, or emulsions in oily or aqueous vehicles, and may contain formulatory agents such as suspending, stabilizing and/or dispersing agents. Alternatively, the nitrites and other ingredients may be in powder form, obtained by aseptic isolation of sterile solid or by lyophilization from solution, for constitution with a suitable vehicle, *e.g.*, sterile, pyrogen-free water, before use.

These formulations can contain pharmaceutically acceptable carriers and vehicles that are available in the art. It is possible, for example, to prepare solutions using one or more organic solvent(s) that is/are acceptable from the physiological standpoint, chosen, in addition to water, from solvents such as acetone, ethanol, isopropyl alcohol, glycol ethers such as the products sold under the name "Dowanol," polyglycols and polyethylene glycols, C_1-C_4 alkyl esters of short-chain acids, ethyl or isopropyl lactate, fatty acid triglycerides such as the products marketed under the name "Miglyol," isopropyl myristate, animal, mineral and vegetable oils and polysiloxanes.

It is possible to add other ingredients such as antioxidants, surfactants, preservatives, film-forming, keratolytic or comedolytic agents, perfumes, flavorings and colorings. Antioxidants such as t-butylhydroquinone, butylated hydroxyanisole, butylated hydroxytoluene and α-tocopherol and its derivatives can be added.

The pharmaceutical formulations of the present invention may include, as optional ingredients, pharmaceutically acceptable carriers, diluents, solubilizing or emulsifying agents, and salts of the type that are available in the art. Examples of such substances include normal saline solutions such as physiologically buffered saline solutions and water. Specific non-limiting examples of the carriers and/or diluents that are useful in the pharmaceutical formulations of the present invention include water and physiologically acceptable buffered saline solutions such as phosphate buffered saline solutions at a pH of about 7.0-8.0.

The nitrites can also be administered via the respiratory tract. Thus, the present invention also provides aerosol pharmaceutical formulations and dosage forms for use in the methods of the invention. In general, such dosage forms include an amount of nitrite effective to treat or prevent the clinical symptoms of a specific condition. Any attenuation, for example a statistically significant attenuation, of one or more symptoms of a condition that has been treated pursuant to the methods of the present invention is considered to be a treatment of such condition and is within the scope of the invention.

For administration by inhalation, the composition may take the form of a dry powder, for example, a powder mix of the nitrite and a suitable powder base such as lactose or starch. The powder composition may be presented in unit dosage form in, for example, capsules or cartridges, or, e.g., gelatin or blister packs from which the powder may be administered with the aid of an inhalator, insufflator, or a metered-dose inhaler (see, for example, the pressurized metered dose inhaler (MDI) and the dry powder inhaler disclosed in Newman, S. P. in Aerosols and the Lung, Clarke, S. W. and Davia, D. eds., pp. 197-224, Butterworths, London, England, 1984).

Nitrites may also be administered in an aqueous solution, for example, when administered in an aerosol or inhaled form. Thus, other aerosol pharmaceutical formulations may include, for example, a physiologically acceptable buffered saline solution. Dry aerosol in the form of finely divided solid compound that is not dissolved or suspended in a liquid is also useful in the practice of the present invention.

For administration to the respiratory tract, for example, the upper (nasal) or lower respiratory tract, by inhalation, the nitrites can be conveniently delivered from a nebulizer or a pressurized pack or other convenient means of delivering an aerosol spray. Pressurized packs may include a suitable propellant such as dichlorodifluoromethane, trichlorofluoromethane, dichlorotetrafluoroethane, carbon dioxide or other suitable gas. In the case of a pressurized aerosol, the dosage unit may be determined by providing a valve to deliver a metered amount. Nebulizers include, but are not limited to, those described in U.S. Patent Nos. 4,624,251; 3,703,173; 3,561,444; and 4,635,627. Aerosol delivery systems of the type

disclosed herein are available from numerous commercial sources including Fisons Corporation (Bedford, Mass.), Schering Corp. (Kenilworth, NJ) and American Pharmoseal Co., (Valencia, CA). For intra-nasal administration, the therapeutic agent may also be administered via nose drops, a liquid spray, such as via a plastic bottle atomizer or metered-dose inhaler. Typical of atomizers are the Mistometer (Wintrop) and the Medihaler (Riker). The nitrites may also be delivered via an ultrasonic delivery system. In some embodiments of the invention, the nitrites may be delivered via an endotracheal tube. In some embodiments of the invention, the nitrites may be delivered via a face mask.

Furthermore, the nitrite may also be used in combination with other therapeutic agents, for example, pain relievers, anti-inflammatory agents, antihistamines, and the like, whether for the conditions described or some other condition.

The present invention further pertains to a packaged pharmaceutical composition such as a kit or other container. The kit or container holds a therapeutically effective amount of a pharmaceutical composition of nitrite and instructions for using the pharmaceutical composition for treating a condition.

The invention is further illustrated by the following non-limiting Example.

Example

Nitrite has vasodilatory properties *in vivo*

Eighteen healthy subjects (9 males, 9 females; age range 21 to 50 years) were enrolled in a physiological study to determine if nitrite is a vasodilator and to examine nitrite's *in vivo* chemistry. Part I of the protocol was designed to measure the normal hemodynamic and metabolic responses to exercise and to inhibition of NO synthesis within the forearm as a control for Part II of the protocol, in which these interventions were performed during nitrite infusion. Initial baseline measurements included a mean blood pressure of 85.6 ± 3.7 mm Hg and forearm blood flow of 4.0 ± 0.3 ml/min per 100 mL tissue (Figure 1A). Repetitive hand-grip forearm exercise increased blood flow approximately 600% over resting values, and significantly decreased ipsilateral venous hemoglobin oxygen saturation, pO_2, and

pH, consistent with increased oxygen consumption and CO_2 generation. Following a 20-minute rest period, repeat hemodynamic measurements showed an approximate 10% higher forearm blood flow, but no change in systemic blood pressure or forearm venous hemoglobin oxygen saturation, pO_2 and pH values compared with the initial baseline values (Figure 1B). The NO synthase inhibitor L-NMMA was then infused into the brachial artery at 8 μmol/min for 5 minutes, significantly reducing forearm blood flow by approximately 30% and significantly reducing venous hemoglobin oxygen saturation, pO_2 and pH values. Repeat forearm exercise during continued L-NMMA infusion increased blood flow, but to a significantly lower peak value compared with exercise alone ($P<0.001$). In addition, hemoglobin oxygen saturation, pO_2 and pH were significantly lower during exercise with L-NMMA than with exercise without regional NO synthase inhibition ($P<0.001$, $P<0.005$ and $P=0.027$, respectively). Mean arterial blood pressure was unchanged during all components of Part I of the protocol.

Figure 1 depicts hemodynamic and metabolic measurements at baseline and during exercise, without (Figure 1A) and with (Figure 1B) inhibition of NO synthesis in 18 subjects. Mean arterial pressure (MAP), forearm blood flow (FBF), and venous oxyhemoglobin saturation, partial pressure of oxygen (pO_2), and pH are shown for all experimental conditions. These interventions and measurements (part I of the protocol) served as a control for Part II of the protocol, in which these interventions were performed during nitrite infusion.

To determine whether nitrite has vasoactivity in humans, in Part II of the protocol sodium nitrite in bicarbonate-buffered normal saline (final concentration 36 μmol/ml) was infused into the brachial arteries of these 18 subjects to achieve an estimated intravascular concentration of approximately 200 μM. (Lauer et al., 2001) Following repeat baseline measurements and infusion of sodium nitrite at 1 mL/min for 5 minutes, nitrite levels in the ipsilateral antecubital vein increased from 3.32 ± 0.32 to 221.82 ± 57.59 μM (Figure 2A). Forearm blood flow increased 175% over resting values; venous hemoglobin oxygen saturation, pO_2 and pH levels significantly increased over pre-infusion values, consistent with increased perfusion of the forearm.

Systemic levels of nitrite were 16 μM as measured in the contralateral arm and were associated with a systemic effect of decreased mean blood pressure of approximately 7 mm Hg. Consistent with immediate NO generation from nitrite during an arterial-to-venous transit, iron-nitrosylated-hemoglobin in the ipsilateral antecubital vein increased from 55.7 ± 11.4 to 693.4 ± 216.9 nM during the nitrite infusion. During forearm exercise with continuation of the nitrite infusion, blood flow increased further, with evidence of metabolic stress by virtue of reduction in forearm venous hemoglobin oxygen saturation, pO_2 and pH levels from baseline values. Venous nitrite levels declined, consistent with increased blood flow to the forearm diluting the concentration of infused nitrite. Despite decreasing forearm nitrite concentrations during exercise, iron-nitrosyl-hemoglobin levels increased. (Figure 2A).

Following cessation of nitrite infusion and substitution of saline as the intra-arterial infusate for 30 minutes, repeat baseline measurements showed persistent elevations in systemic levels of nitrite, iron-nitrosyl-hemoglobin and methemoglobin (Figure 2B) over values obtained prior to the infusion of nitrite almost one hour before. In addition, persistence of a vasodilator effect was also apparent, as forearm blood flow was significantly higher (4.79 ± 0.37 versus 3.94 ± 0.38 mL/min per 100 mL tissue, P=0.003) and systemic blood pressure significantly lower (82.1± 3.7 versus 89.2 ± 3.5 mm Hg, P=0.002) than initial pre-nitrite infusion values. During re-infusion into the brachial artery of sodium nitrite 36 μmol/ml, combined with L-NMMA 8 μmol/min in order to again inhibit regional synthesis of NO, similar vasodilator effects of nitrite on resting and exercise forearm blood flow were seen as during nitrite infusion without L-NMMA (Figure 2B). This stands in contrast to the vasoconstrictor effect of NO synthase inhibition with L-NMMA observed in Part I of the protocol (Figure 1B). Venous nitrite and iron-nitrosyl-hemoglobin levels followed similar patterns during NO inhibition as during the initial nitrite infusion.

Figure 2 depicts the effects of infusion of sodium nitrite ($NaNO_2$) in bicarbonate-buffered normal saline (0.9%; final concentration 36 μmol/ml) into the brachial arteries of 18 healthy subjects at 1 ml/min for 5 minutes at baseline and

continued during exercise. Figure 2A depicts the effects without inhibition of NO synthesis. Figure 2B depicts the effects with inhibition of NO synthesis. Values for mean arterial blood pressure (MAP), forearm blood flow (FBF), venous oxyhemoglobin saturation, partial pressure of oxygen (pO_2) and pH, venous nitrite, venous iron-nitrosyl-hemoglobin and venous methemoglobin are shown for all experimental interventions.

As a test of the physiological relevance of vascular nitrite as a vasodilator, nitrite concentrations were decreased by 2-logs to 400 nmol/mL. An infusion of 1 mL/min for five minutes in 10 subjects significantly increased forearm blood flow in all ten subjects from 3.49 ± 0.24 to 4.51 ± 0.33 ml/min per 100 mL tissue (Figure 3A; P=0.0006). Blood flow significantly increased at rest and during NO synthase inhibition with and without exercise (Figure 3B; $P<0.05$ during all conditions). Mean venous nitrite levels increased from 176 ± 17 nM to 2564 ± 462 nM following a five-minute infusion and exercise venous nitrite levels decreased to 909 ± 113 nM (secondary to dilutional effects of increased flow during exercise; Figure 3C). Again, the vasodilator effects of nitrite were paralleled with an observed formation of both iron-nitrosyl-hemoglobin and S-nitroso-hemoglobin across the forearm circulation (Figure 3D; described below). These data indicate that basal levels of nitrite, from 150-1000 nM in plasma to 10,000 nM in vascular tissue, contribute to resting vascular tone and hypoxic vasodilation.

Figure 3 depicts the effects of infusion of low-dose sodium nitrite in bicarbonate-buffered normal saline into the brachial arteries of 10 healthy subjects at baseline and during exercise, without and with inhibition of NO synthesis. Figure 3A depicts forearm blood flow at baseline and following a five-minute in fusion of $NaNO_2$ (0.36 μmol/ml in 0.9% saline, infused at 1 ml/min). Figure 3B depicts forearm blood flow with and without low-dose nitrite infusion at baseline and during L-NMMA infusion with and without exercise stress. Figure 3C depicts venous levels of nitrite from the forearm circulation at the time of blood flow measurements. Figure 3D depicts venous levels of S-nitroso-hemoglobin (S-NO) and iron-nitrosyl-hemoglobin (Hb-NO) at baseline and following nitrite infusion during exercise stress.

The vasodilatory property of nitrite during basal blood flow conditions, when tissue pO_2 and pH are not exceedingly low, was unexpected. These results indicate that the previously hypothesized mechanisms for nitrite reduction, nitrite disproportionation and xanthine oxidoreductase activity, both of which require extremely low pO_2 and pH values not typically encountered in normal physiology, are complemented *in vivo* by additional factors that serve to catalyze nitrite reduction. While ascorbic acid and other reductants, present in abundance in blood, can provide necessary electrons for nitrous acid reduction, such that the reaction might occur at physiologically attainable pH levels, it is herein reported that deoxyhemoglobin effectively reduces nitrite to NO, within one half-circulatory time. This mechanism provides a graded production of NO along the physiological oxygen gradient, tightly regulated by hemoglobin oxygen desaturation.

Before and during nitrite infusions, blood was drawn from both the brachial artery and antecubital vein and the whole blood immediately (at the bedside to eliminate processing time) lysed 1:10 in an NO-hemoglobin "stabilization solution" and the iron-nitrosyl-hemoglobin and S-nitroso-hemoglobin content determined by tri-iodide-based reductive chemiluminescence and electron paramagnetic resonance spectroscopy as described in Methods. The baseline levels of S-nitroso-hemoglobin and iron-nitrosyl-hemoglobin were at the limits of detection (<50 nM or 0.0005% NO per heme) with no artery-to-vein gradients. Following nitrite infusion in Part II of the protocol venous levels of both iron-nitrosyl-hemoglobin and S-nitroso-hemoglobin rose strikingly (Figure 4A). The formation of both NO-hemoglobin adducts occurred across the vascular bed, a half-circulatory time of less than 10 seconds. The rate of NO formation, measured as iron-nitrosyl and S-nitroso-hemoglobin and quantified by subtraction of the arterial from the venous levels with the difference being multiplied by blood flow, increased greatly during exercise, despite a significant decrease in the venous concentration of nitrite secondary to increasing blood flow diluting the regional nitrite concentration (Figure 4A; P=0.006 for iron-nitrosyl-hemoglobin and P=0.02 for S-nitroso-hemoglobin by repeated measures ANOVA).

Figure 4A depicts formation of iron-nitrosyl-hemoglobin (black squares) and S-nitroso-hemoglobin (red circles) during nitrite infusion at baseline, during nitrite infusion and during nitrite infusion with exercise, quantified by subtraction of the arterial from the venous levels and multiplying the result by blood flow. The formation of both NO-hemoglobin adducts was inversely correlated with hemoglobin-oxygen saturation in the human circulation during nitrite infusion (for iron-nitrosyl-hemoglobin $r=-0.7$, $p<0.0001$, for S-nitroso-hemoglobin $r=-0.45$, $p=0.04$) (Figure 4B). Hemoglobin oxygen saturation was measured from the antecubital vein by co-oximetry. Asterix in all figures signify $P<0.05$ by paired t test or repeated measures analysis of variance.

To determine whether free NO radical can form from the reaction of nitrite and deoxyhemoglobin, 100 and 200 μM nitrite was reacted with deoxygenated erythrocytes (5 mL volume containing a total of 660 and 1000 μM in heme) in a light protected reaction vessel purged with helium in-line with a chemiluminescent NO analyzer (Seivers, Boulder, CO.). As shown in Figure 5A and 5B, the injection of nitrite into a solution of deoxygenated erythrocytes resulted in the liberation of NO into the gas phase. There was no release from nitrite in buffer control under the same conditions, and significantly less NO was released upon nitrite addition to oxygenated erythrocytes (21% and 100% oxygen). The observed rate (determined by the assessment of the area under the curve of increased steady-state NO generation following nitrite injection calculated over 120 seconds) of NO production in the 5 mL reaction volume was consistent with 47 pM NO production per second (corresponding to an estimated 300 to 500 pM NO production per second in whole blood). While NO formation rates in this experimental system may not be extrapolated to rates of NO formation *in vivo*, the experiments are consistent with two important concepts: 1) A fraction of free NO can escape auto-capture by the remaining heme groups; this is likely only possible because nitrite is only converted to NO by reaction with deoxyhemoglobin and its "leaving group" is the met(ferric)heme protein which will limit scavenging and inactivation of NO. (Doyle et al., 1981); and 2) The rate of NO production is increased under anaerobic conditions, consistent with a nitrite-deoxyhemoglobin reaction.

Methods

Human subjects protocol.

The protocol was approved by the Institutional Review Board of the National Heart, Lung and Blood Institute, and informed consent was obtained from all volunteer subjects. Nine men and nine women, with an average age of 33 years (range 21 - 50 years), participated in the study. An additional 10 subjects returned three-six months later for a second series of experiments with low dose nitrite infusion. Volunteers had a normal hemoglobin concentration, and all were in excellent general health without risk factors for endothelial dysfunction (fasting blood sugar >120 mg/dL, low-density lipoprotein cholesterol >130 mg/dL, blood pressure >145/95 mmHg, smoking within two years, cardiovascular disease, peripheral vascular disease, coagulopathy, or any other disease predisposing to vasculitis or Raynaud's phenomenon). Subjects with G6PD deficiency, known cytochrome B5 deficiency or a baseline methemoglobin level > 1% were excluded (no screened patients met these exclusion criteria). Lactating and pregnant females were excluded (one patient with positive HCG levels was excluded). No volunteer subject was allowed to take any medication (oral contraceptive agents allowed), vitamin supplements, herbal preparations, nutriceuticals or other "alternative therapies" for at least one month prior to study and were not be allowed to take aspirin for one week prior to study.

Forearm blood flow measurements

Brachial artery and antecubital vein catheters were placed into the arm, with the intra-arterial catheter connected to a pressure transducer for blood pressure measurements and an infusion pump delivering normal saline at 1 mL/min. After 20 minutes of rest, baseline arterial and venous blood samples were obtained and forearm blood flow measurements were made by strain gauge venous-occlusion plethysmography, as previously reported. (Panza et al., 1993) A series of 7 blood flow measurements were averaged for each blood flow determination. A series of

measurements termed Parts I and II were performed in randomized order to minimize a time effect on the forearm blood flow response during nitrite infusion.

Measurement of blood flow and forearm nitrite extraction during NO blockade and repetitive exercise

Part I: Following 20 minutes of 0.9% NaCl (saline) solution infusion at 1 mL/min into the brachial artery, arterial and venous blood samples were obtained for the assays described below and forearm blood flow measured. Exercise was performed by repetitive hand-grip at one-third of the predetermined maximum grip strength using a hand-grip dynamometer (Technical Products Co.). (Gladwin et al., 2000; Cannon, 2001) Each contraction lasted for 10 seconds followed by relaxation for 5 seconds. Following 5 minutes of exercise, forearm blood flow measurements were obtained during relaxation phases of exercise, and arterial and venous samples collected. Following a 20-minute rest period with continued infusion of saline into the brachial artery, repeated baseline blood samples and forearm blood flow measurements were obtained. L-NMMA was then infused at a rate of 1 mL/min (8 μmol/min) into the brachial artery. Following 5 minutes of L-NMMA infusion, forearm blood flow was measured, and arterial and venous blood samples obtained. Forearm exercise was then initiated in that arm during continued L-NMMA infusion. Forearm blood flow was measured and blood samples obtained after 5 minutes of exercise during continued L-NMMA infusion (Figure 1).

Part II: After a 30 minute rest period with continued infusion of saline, baseline measurements were obtained, the saline infusion was then stopped, and infusion of nitrite ($NaNO_2$ 36 μmol/ml in 0.9% saline) at 1 ml/min was started. Sodium nitrite for use in humans was obtained from Hope Pharmaceuticals (300 mg in 10 ml water) and 286 mg was diluted in 100 ml 0.9% saline by the Pharmaceutical Development Service to a final concentration of 36 μmol/ml. For the final 9 subjects studied, 0.01-0.03 mM sodium bicarbonate was added to the normal saline, so as to titrate pH to 7.0-7.4. The nitrite solution was light protected and nitrite levels and free NO gas in solution measured by reductive chemiluminescence after all experiments. (Gladwin, 2002) Only 50.5 ± 40.5 nM

NO was present in nitrite solutions and was unaffected by bicarbonate buffering. There was no correlation between NO levels in nitrite solutions and blood flow effects of nitrite (r = -0.23; P=0.55). After 5 minutes of nitrite infusion, forearm blood flow measurements and blood samples were obtained, with brief interruption of the nitrite infusion to obtain the arterial sample. With continued nitrite infusion, exercise was performed as described previously, with forearm blood flow measurements and blood samples obtained as described above. The nitrite infusion was stopped and saline infusion re-started during the subsequent 30-minute rest period. Following second baseline measurements, the nitrite infusion was re-initiated, along with L-NMMA at 8 μmol/min. Five minutes later, forearm blood flow measurements were performed and blood samples obtained followed by 5 minutes of exercise with continuation of nitrite and L-NMMA infusions. Final forearm blood flow measurements and blood samples obtained. At all time points during part II, blood samples were obtained from the contralateral arm antecubital vein for determination of methemoglobin and systemic levels of NO-modified hemoglobin (Figure 2, 3, and 4). The total dose of sodium nitrite infused was 36 μmol/min x 15 minutes x 2 infusions = 1.08 mmol = 75 mg (MW $NaNO_2$ = 69).

In additional studies in 10 subjects the same stages of Parts I and II protocol were followed with infusion of low dose nitrite ($NaNO_2$ 0.36 μmol/ml in 0.9% saline, infused at 1 ml/min).

Arterial and venous pH, pO_2, and pCO_2, were measured at the bedside using the i-STAT system (i-STAT Corporation, East Windsor, NJ) and methemoglobin concentration and hemoglobin oxygen saturation measured by co-oximetry.

Measurement of red blood cell S-nitroso-hemoglobin and iron-nitrosyl-hemoglobin.

S-nitroso-hemoglobin is unstable in the reductive red blood cell environment and rapidly decays in a temperature and redox dependent fashion, independent of oxygen tension. (Gladwin, 2002) to stabilize the S-nitroso-hemoglobin for measurement, the red blood cell must be rapidly oxidized with ferricyanide. Before and during nitrite infusions, blood was drawn from both the brachial artery and antecubital vein and the whole blood immediately (at the bedside to eliminate

processing time) lysed 1:10 in an NO-hemoglobin "stabilization solution" of PBS containing 1% NP-40 (to solubilize membranes), 8 mM NEM (to bind free thiol and prevent artefactual S-nitrosation), 0.1 mM DTPA (to chelate trace copper), and 4 mM ferricyanide and cyanide (to stabilize S-nitrosohemoglobin and prevent artefactual ex-vivo iron-nitrosylation during processing). The samples were desalted across a 9.5 mL bed volume Sephadex G25 column to eliminate nitrite and excess reagents and partially purify hemoglobin (99% hemoglobin preparation). The hemoglobin fraction was quantified by the method of Drabkin, and hemoglobin fractions reacted with and without mercuric chloride (1:5 $HgCl_2$:heme ratio- used to differentiate S-nitrosothiol which is mercury labile vs iron-nitrosyl which is mercury stable) and then in 0.1 M HCL/0.5%sulfanilamide (to eliminate residual nitrite (Marley et al., 2000)). The samples were then injected into a solution of tri-iodide (I_3^-) in-line with a chemiluminescent nitric oxide analyzer (Sievers, Model 280 NO analyzer, Boulder, CO). The mercury stable peak represents iron-nitrosyl-hemoglobin. This assay is sensitive and specific for both S-nitroso-hemoglobin and iron-nitrosyl-hemoglobin to 5 nM in whole blood (0.00005% SNO per heme). (Gladwin, 2002)

Analysis was initially performed using red blood cell pellet, however, despite placing the sample in ice and immediately separating plasma from erythrocyte pellet, NO formed in the venous blood *ex vivo*. To measure the true *in vivo* levels, whole blood was mixed at the bedside 1:10 in the "NO-hemoglobin stabilization solution". Plasma S-nitroso-albumin formation was negligible during nitrite infusion so this bedside whole blood assay was used to limit processing time and thus more accurately characterize the *in vivo* chemistry. In a series of validation experiments, both S-nitroso-hemoglobin and iron-nitrosyl-hemoglobin were stable in the "NO-hemoglobin stabilization solution" for 20 minutes at room temperature with no artifactual formation or decay of NO-modified species (n=6; data not shown).

Chemiluminescent detection of NO gas released from deoxyhemoglobin and deoxygenated erythrocytes following nitrite addition.

To determine whether free NO radical can form from the reaction of nitrite and deoxyhemoglobin, 100 and 200 μM nitrite was mixed with 5 mL of 660 and 1000 μM deoxygenated erythrocytes in a light protected reaction vessel purged with helium or oxygen (both 21% and 100%) in-line with a chemiluminescent NO analyzer (Seivers, Boulder, CO.). After allowing equilibration for 5 minutes, nitrite was injected and the rate of NO production measured. Nitrite was injected into PBS as a control and into 100 μM hemoglobin to control for the hemolysis in the 660 and 1000 μM deoxygenated erythrocyte solutions. At the end of all experiments the visible absorption spectra of the supernatant and erythrocyte reaction mixture was analyzed and hemoglobin composition deconvoluted using a least-squares algorithm. There was less than 100 μM hemolysis in the system, no hemoglobin denaturation, and significant formation of iron-nitrosyl-hemoglobin. The NO production from erythrocyte suspensions exceeded that produced from the hemolysate control, consistent with NO export from the erythrocyte.

Statistical analysis.

An a priori sample size calculation determined that 18 subjects would be necessary for the study to detect a 25% improvement in forearm blood flow during nitrite infusion when forearm NO synthesis had been inhibited by L-NMMA compared with normal saline infusion control values (alpha=0.05, power=0.80). Two-sided P values were calculated by paired t-test for the pair-wise comparisons between baseline and L-NMMA infusion values, between baseline and exercise values, and between nitrite and saline control values at comparable time-points of the study. Repeated measures ANOVA was performed for artery-to-vein gradients of NO species during basal, L-NMMA infusion, and exercise conditions. Measurements shown are mean ± SEM.

All publications, patents and patent applications cited herein are herein incorporated by reference.

While in the foregoing specification this invention has been described in relation to certain preferred embodiments thereof, and many details have been set forth for purposes of illustration, it will be apparent to those skilled in the art that the invention is susceptible to additional embodiments, and that certain of the details described herein may be varied considerably without departing from the basic principles of the invention.

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The following statements of the invention are intended to characterize possible elements of the invention according to the foregoing description given in the specification. Because this application is a provisional application, these statements may be changed upon preparation and filing of a nonprovisional application. Such changes are not intended to affect the scope of equivalents according to the claims issuing from the nonprovisional application, if such changes occur. According to 35 U.S.C. § 111(b), claims are not required for a provisional application. Consequently, the statements of the invention cannot be interpreted to be claims pursuant to 35 U.S.C. § 112.

Formal Claim

1. A method for decreasing a patient's blood pressure, comprising administering to the patient sodium nitrite at about 36 μmoles per minute into the forearm brachial artery.

Statements of the Invention

1. A method for decreasing a patient's blood pressure, comprising administering to the patient an effective amount of pharmaceutically-acceptable nitrite so as to decrease the patient's blood pressure.

2. A method for treating a patient having a condition associated with elevated blood pressure, comprising administering to the patient an effective amount of pharmaceutically-acceptable nitrite so as to treat at least one vascular complication associated with the elevated blood pressure.

3. A method for treating a patient having a hemolytic condition, comprising administering to the patient an effective amount of pharmaceutically-acceptable nitrite so as to treat at least one vascular complication associated with the hemolytic condition.

4. The method of statement 2 or 3, wherein the vascular complication is one or more selected from the group consisting of pulmonary hypertension, systemic hypertension, cutaneous ulceration, acute renal failure, chronic renal failure, intravascular thrombosis, an ischemic central nervous system event, and death.

5. The method of statement 2, 3, or 4, wherein the hemolytic condition is one or more selected from the group consisting of sickle cell anemia, thalassemia, hemoglobin C disease, hemoglobin SC disease, sickle thalassemia, hereditary spherocytosis, hereditary elliptocytosis, hereditary ovalcytosis, glucose-6-phosphate

deficiency and other red blood cell enzyme deficiencies, paroxysmal nocturnal hemoglobinuria (PNH), paroxysmal cold hemoglobinuria (PCH), thrombotic thrombocytopenic purpura/hemolytic uremic syndrome (TTP/HUS), idiopathic autoimmune hemolytic anemia, drug-induced immune hemolytic anemia, secondary immune hemolytic anemia, non-immune hemolytic anemia caused by chemical or physical agents, malaria, falciparum malaria, bartonellosis, babesiosis, clostridial infection, severe haemophilus influenzae type b infection, extensive burns, transfusion reaction, ryabdomyolysis (myoglobinemia), transfusion of aged blood, transfusion of hemoglobin, transfusion of red blood cells, cardiopulmonary bypass, coronary disease, cardiac ischemia syndrome, angina, iatrogenic hemolysis, angioplasty, myocardial ischemia, tissue ischemia, hemolysis caused by intravascular devices, and hemodialysis.

6. A method for treating a patient having a condition associated with decreased blood flow to a tissue, comprising administering to the patient an effective amount of pharmaceutically-acceptable nitrite so as to increase blood flow to the tissue of the patient.

7. The method of statement 6, wherein the decreased blood flow to the tissue is caused directly or indirectly by at least one condition selected from the group consisting of: sickle cell anemia, thalassemia, hemoglobin C disease, hemoglobin SC disease, sickle thalassemia, hereditary spherocytosis, hereditary elliptocytosis, hereditary ovalcytosis, glucose-6-phosphate deficiency and other red blood cell enzyme deficiencies, paroxysmal nocturnal hemoglobinuria (PNH), paroxysmal cold hemoglobinuria (PCH), thrombotic thrombocytopenic purpura/hemolytic uremic syndrome (TTP/HUS), idiopathic autoimmune hemolytic anemia, drug-induced immune hemolytic anemia, secondary immune hemolytic anemia, non-immune hemolytic anemia caused by chemical or physical agents, malaria, falciparum malaria, bartonellosis, babesiosis, clostridial infection, severe haemophilus influenzae type b infection, extensive burns, transfusion reaction, ryabdomyolysis (myoglobinemia), transfusion of aged blood, transfusion of

hemoglobin, transfusion of red blood cells, cardiopulmonary bypass, coronary disease, cardiac ischemia syndrome, angina, iatrogenic hemolysis, angioplasty, myocardial ischemia, tissue ischemia, hemolysis caused by intravascular devices, hemodialysis, pulmonary hypertension, systemic hypertension, cutaneous ulceration, acute renal failure, chronic renal failure, intravascular thrombosis, and an ischemic central nervous system event.

8. The method of statement 7, wherein the tissue is an ischemic tissue.

9. The method of statements 7 or 8, wherein the tissue is one or more tissues selected from the group consisting of neuronal tissue, bowel tissue, intestinal tissue, limb tissue, or cardiac tissue.

10. The method of any of statements 1-9, wherein the administration of the nitrite is parenteral, oral, bucal, rectal, *ex vivo*, or intraocular.

11. The method of any of statements 1-9, wherein the administration of the nitrite is peritoneal, intravenous, intraarterial, subcutaneous, inhaled, intramuscular, or into a cardiopulmonary bypass circuit.

12. The method of any of statements 1-11, wherein the nitrite is administered as a pharmaceutically-acceptable salt of nitrite.

13. The method of any one of statements 1-12, wherein the nitrite is administered as sodium nitrite.

14. The method of any one of statements 1-13, wherein the nitrite is administered in combination with at least one additional agent.

15. The method of statement 14, wherein the additional agent is one or more selected from the list consisting of penicillin, hydroxyurea, butyrate, clotrimazole, arginine, or a phosphodiesterase inhibitor.

16. The method of statement 15, wherein the phosphodiesterase inhibitor is sildenafil.

17. The method of any one of statements 1-16, wherein the patient is a mammal.

18. The method of any one of statements 1-17, wherein the patient is a human.

19. A method for treating a patient having a condition associated with elevated blood pressure in the lungs, comprising administering to the patient an effective amount of pharmaceutically-acceptable nitrite.

20. The method of statement 19, wherein the patient has neonatal pulmonary hypertension.

21. The method of statement 19, wherein the patient has primary and/or secondary pulmonary hypertension.

22. The method of any of statements 19-21, wherein the nitrite is nebulized.

ABSTRACT

It has been surprisingly discovered that administration of nitrite to patients causes a reduction in blood pressure and an increase in blood flow to tissues, for example, to tissues in regions of low oxygen tension. This discovery provides useful treatments to regulate a patient's blood pressure and blood flow, for example, by the administration of nitrite salts.

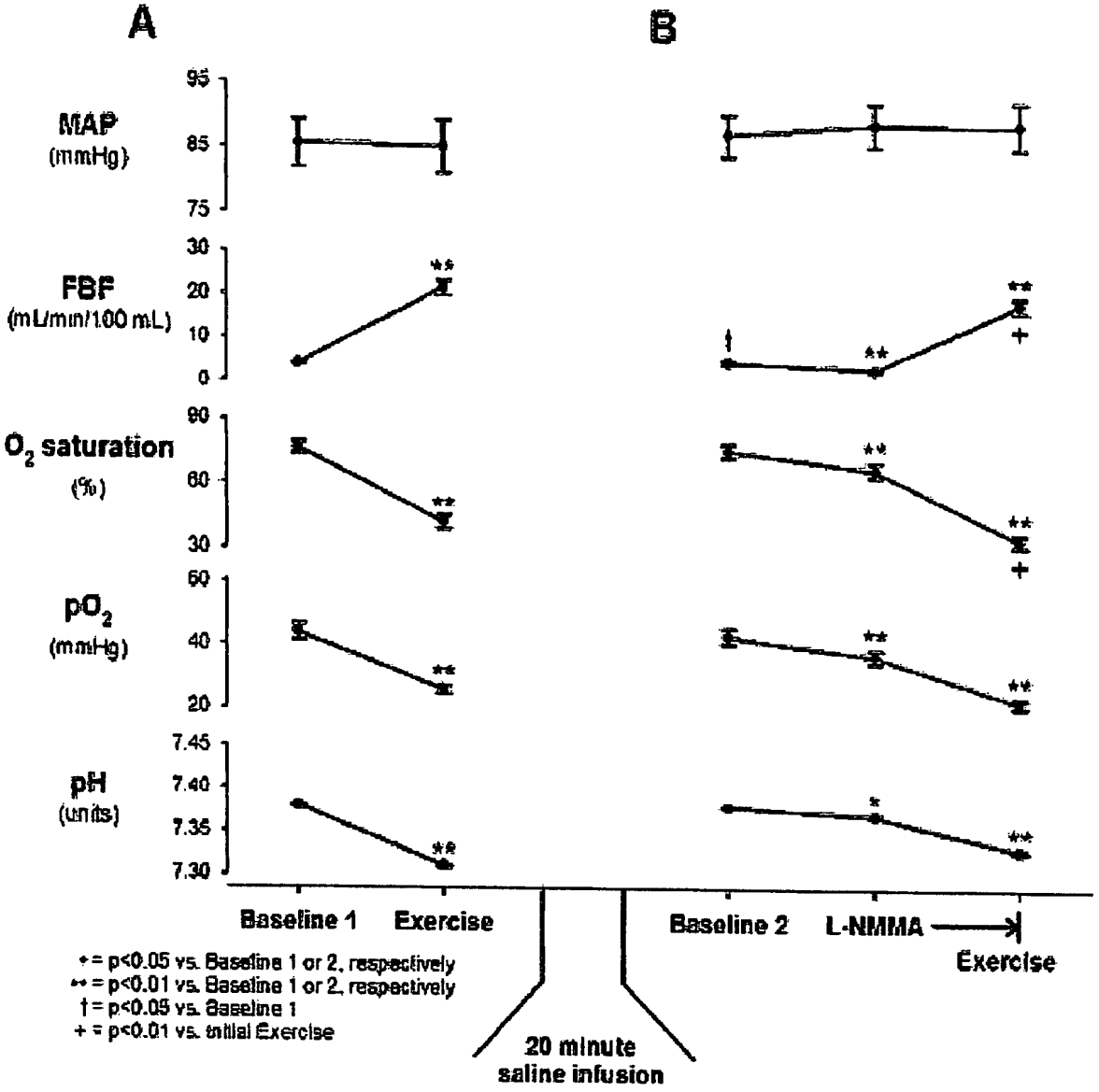
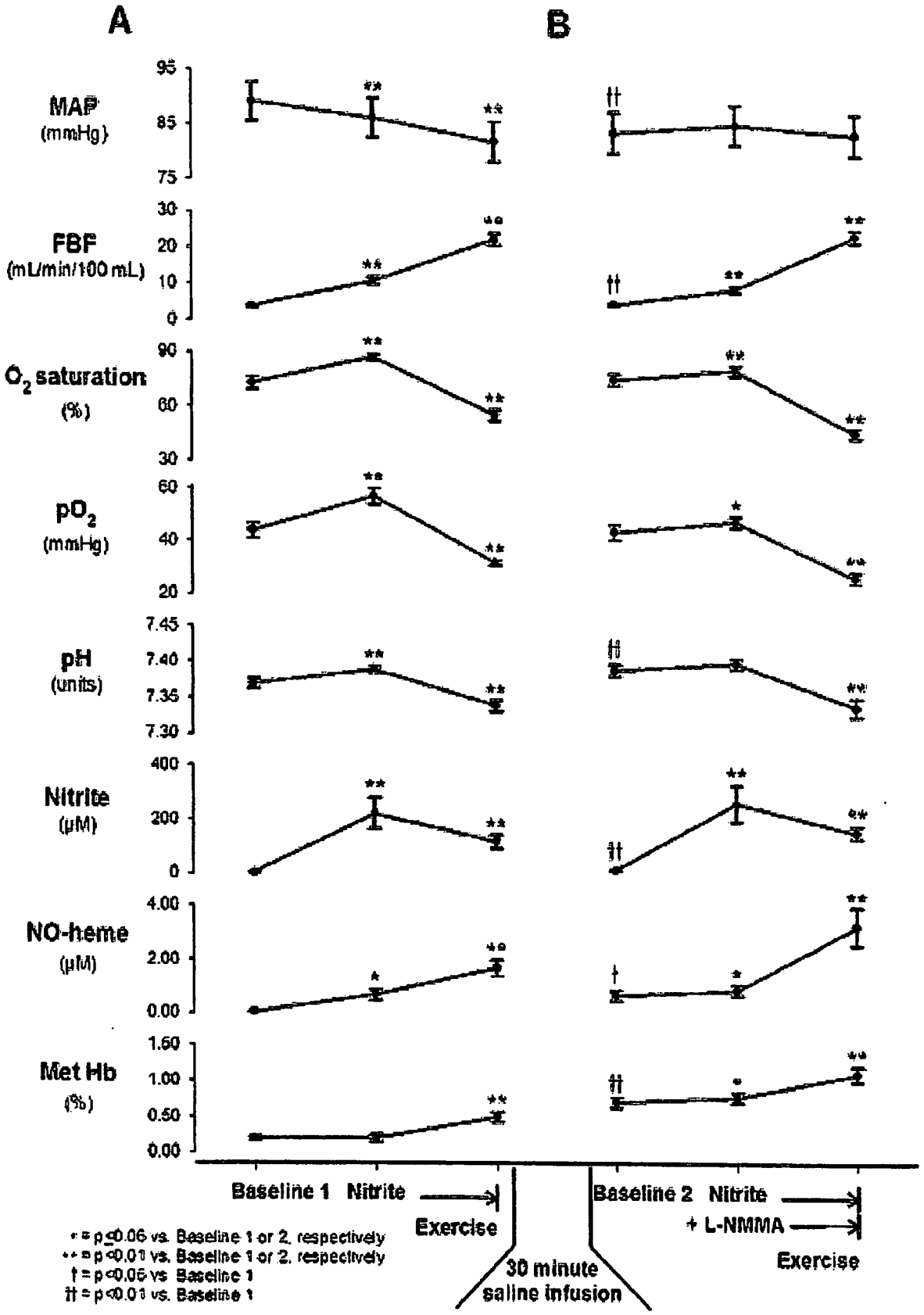
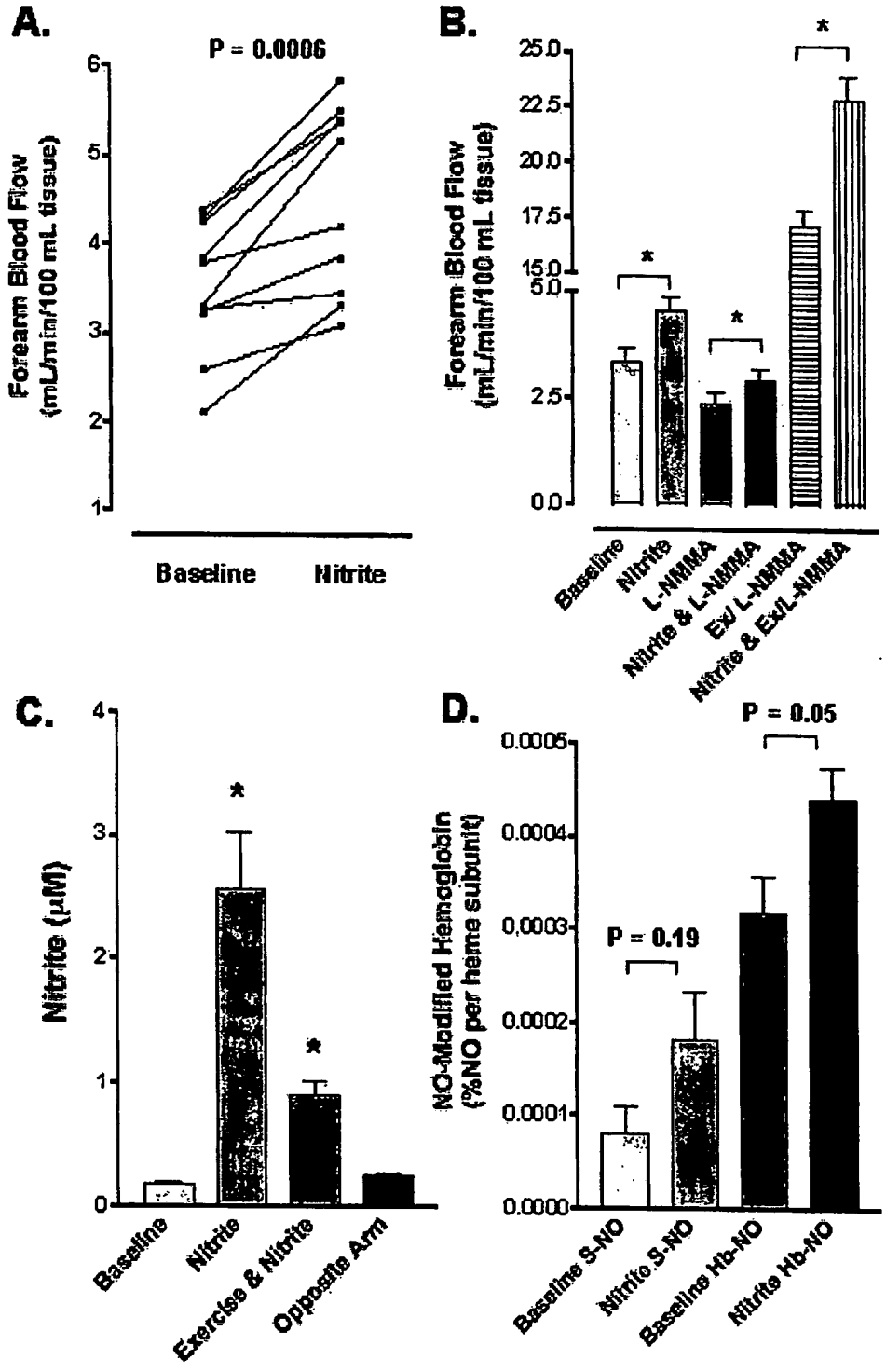

A
B
MAP
(mmHg)
95
85
75
FBF
(mL/min/100 mL)
30
20
10
0
O_2 saturation
(%)
90
60
30
pO_2
(mmHg)
60
40
20
pH
(units)
7.45
7.40
7.35
7.30
Baseline 1
Exercise
Baseline 2
L-NMMA
Exercise
* = p<0.05 vs. Baseline 1 or 2, respectively
** = p<0.01 vs. Baseline 1 or 2, respectively
† = p<0.05 vs. Baseline 1
+ = p<0.01 vs. initial Exercise
20 minute
saline infusion

Fig. 1


A
B
MAP
(mmHg)
95
85
75
FBF
(mL/min/100 mL)
30
20
10
0
O_2 saturation
(%)
90
60
30
pO_2
(mmHg)
60
40
20
pH
(units)
7.45
7.40
7.35
7.30
Nitrite
(μM)
400
200
0
NO-heme
(μM)
4.00
2.00
0.00
Met Hb
(%)
1.50
1.00
0.50
0.00
Baseline 1
Nitrite
Exercise
30 minute
saline infusion
Baseline 2
Nitrite
+ L-NMMA
Exercise
* = p≤0.06 vs. Baseline 1 or 2, respectively
** = p<0.01 vs. Baseline 1 or 2, respectively
† = p<0.06 vs. Baseline 1
†† = p<0.01 vs. Baseline 1

F16.2


A.
P = 0.0006
Forearm Blood Flow (mL/min/100 mL tissue)
Baseline
Nitrite
B.
Forearm Blood Flow (mL/min/100 mL tissue)
Baseline
Nitrite
L-NMMA
Nitrite & L-NMMA
Ex/ L-NMMA
Nitrite & Ex/L-NMMA
C.
Nitrite (μM)
Baseline
Nitrite
Exercise & Nitrite
Opposite Arm
D.
P = 0.05
P = 0.19
NO-Modified Hemoglobin (%NO per heme subunit)
Baseline S-NO
Nitrite S-NO
Baseline Hb-NO
Nitrite Hb-NO

F16. 3

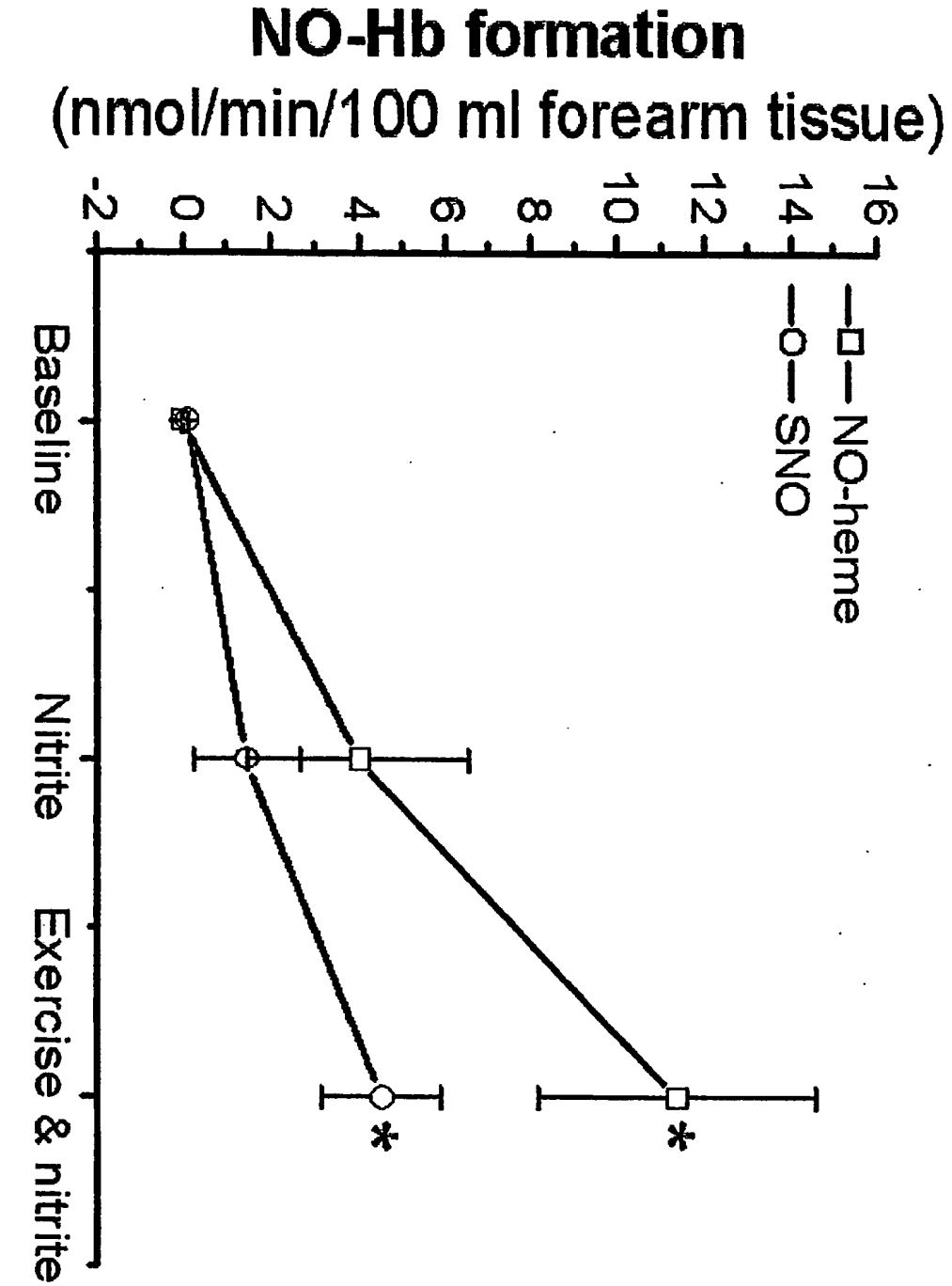

NO-Hb formation
(nmol/min/100 ml forearm tissue)
-2
0
2
4
6
8
10
12
14
16
NO-heme
SNO
Baseline
Nitrite
Exercise & nitrite
*
*

Fig. 4A

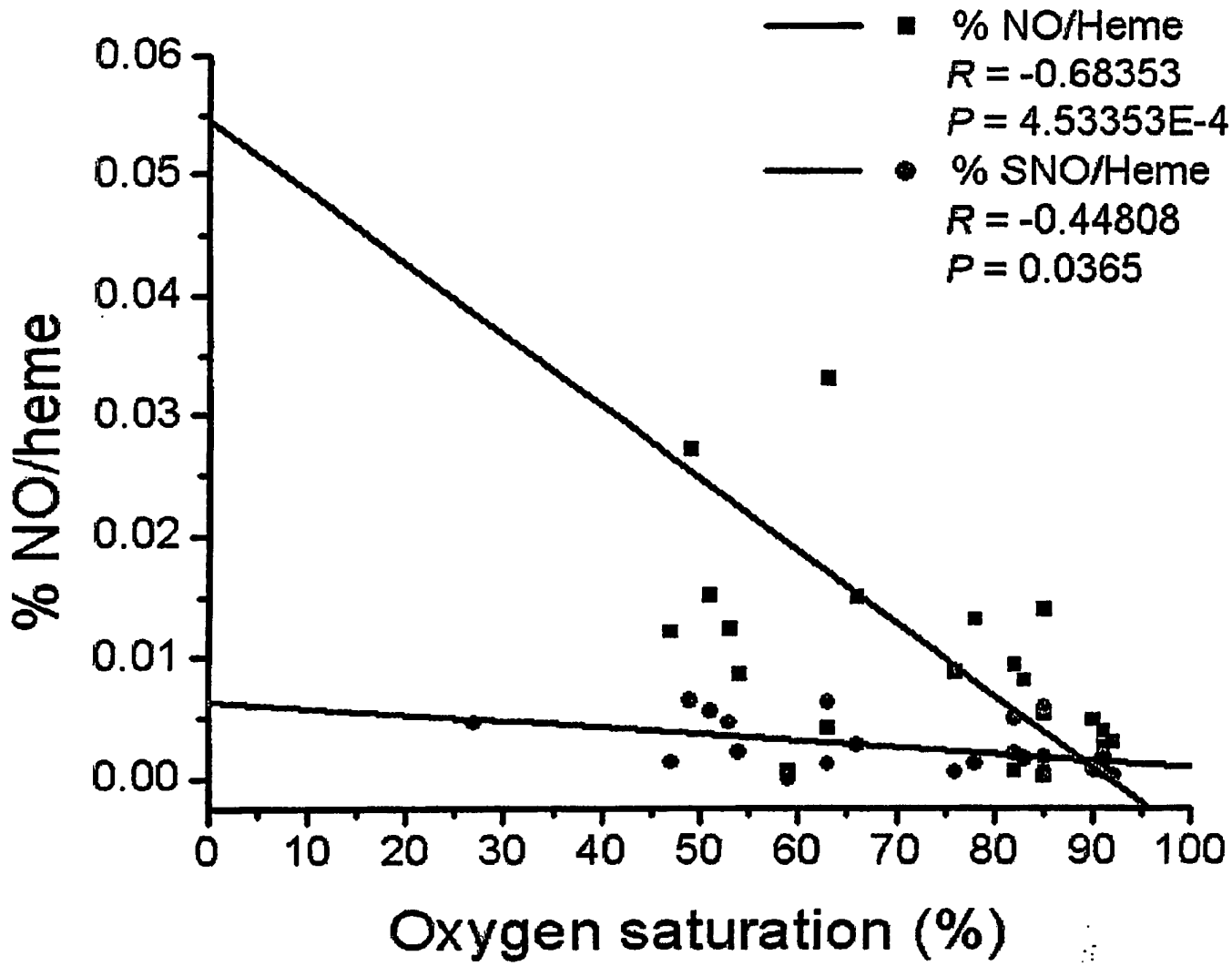

% NO/Heme
R = -0.68353
P = 4.53353E-4
% SNO/Heme
R = -0.44808
P = 0.0365
% NO/heme
0.06
0.05
0.04
0.03
0.02
0.01
0.00
0
10
20
30
40
50
60
70
80
90
100
Oxygen saturation (%)

FIG. 4B

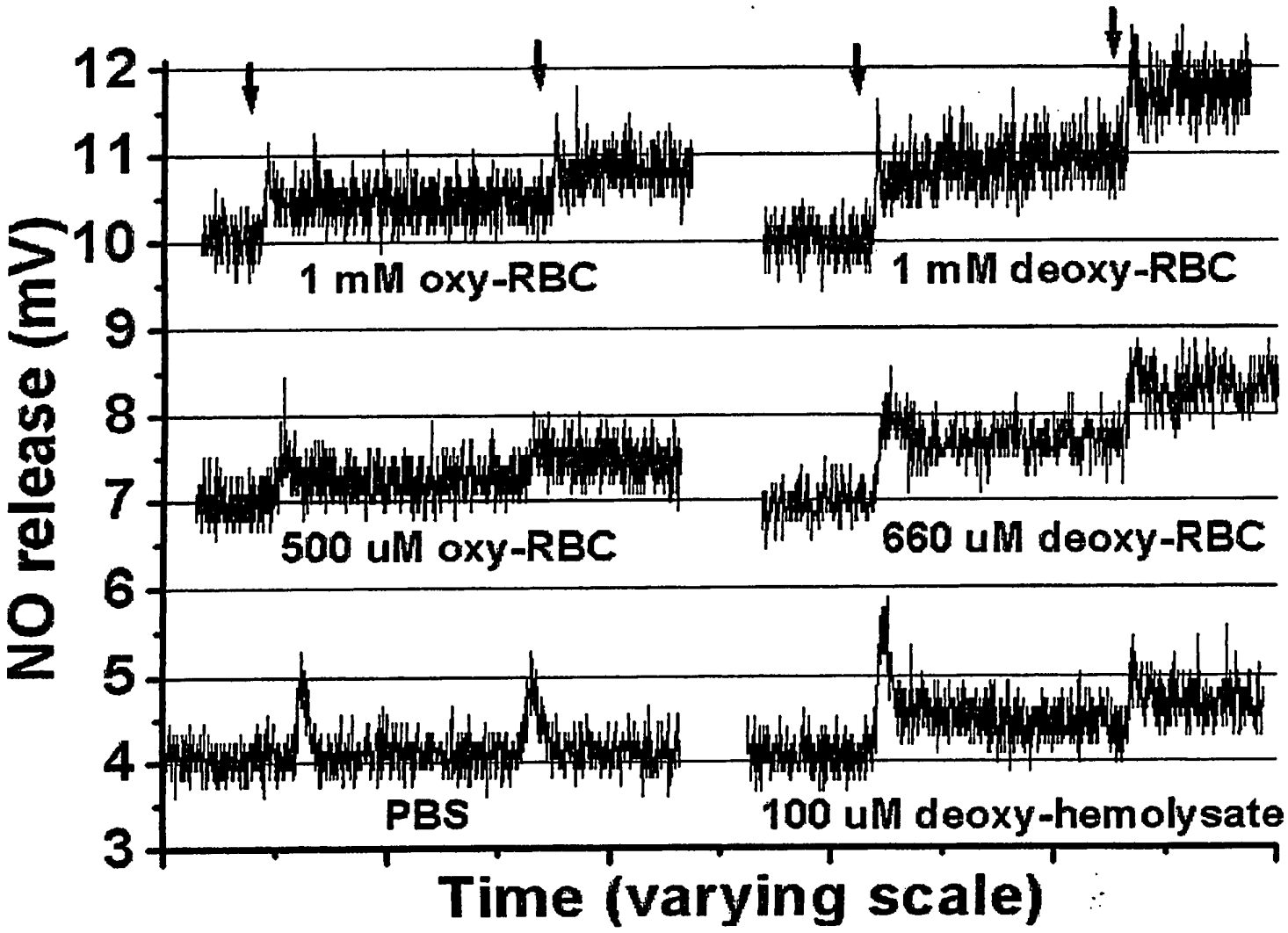

NO release (mV)
12
11
10
9
8
7
6
5
4
3
1 mM oxy-RBC
1 mM deoxy-RBC
500 uM oxy-RBC
660 uM deoxy-RBC
PBS
100 uM deoxy-hemolysate
Time (varying scale)

Fig. 5A

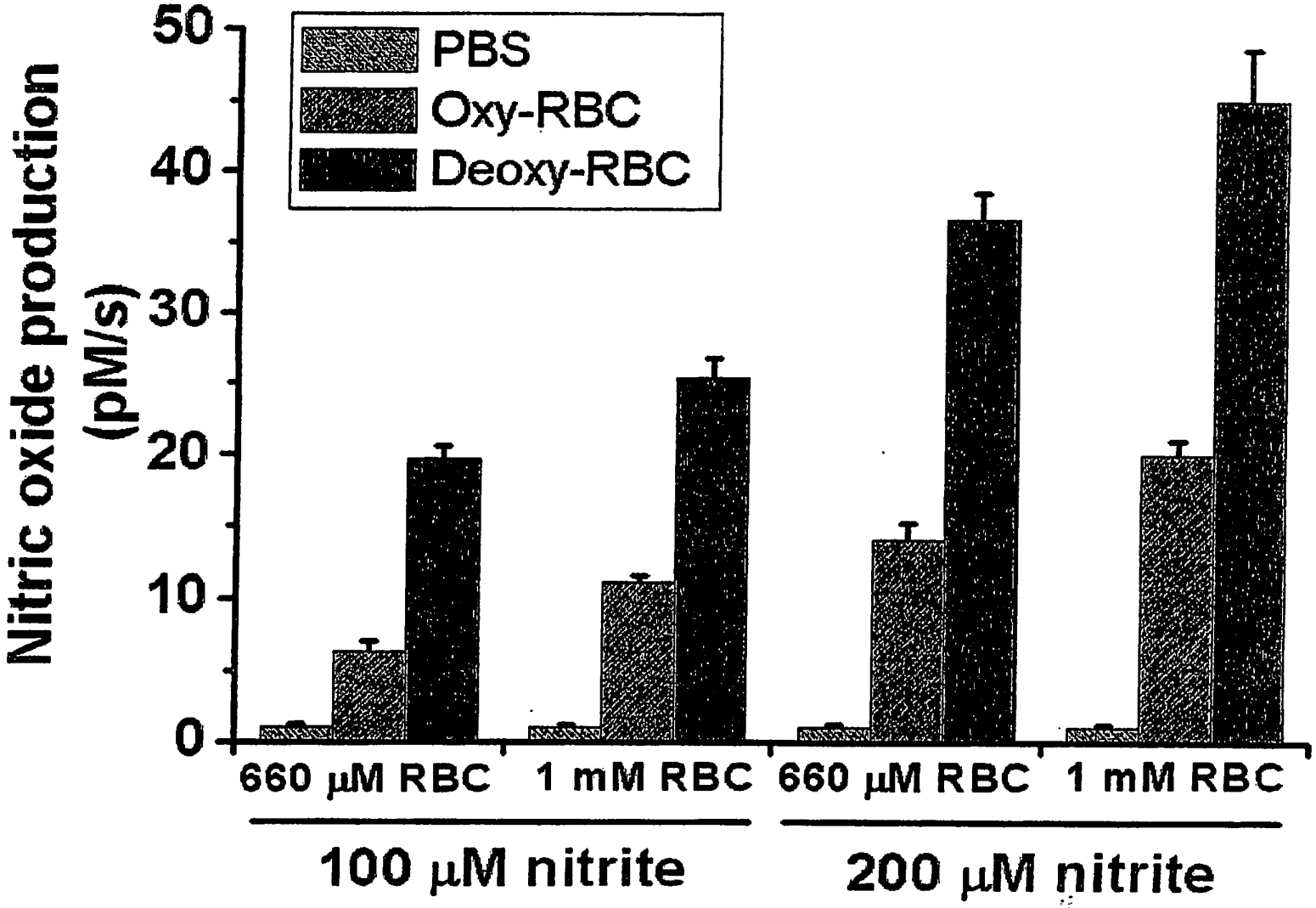

Nitric oxide production (pM/s)
50
40
30
20
10
0
PBS
Oxy-RBC
Deoxy-RBC
660 μM RBC
1 mM RBC
660 μM RBC
1 mM RBC
100 μM nitrite
200 μM nitrite

Fig. 5B

Attorney Docket No.1662.026PV;

SCHWEGMAN ■ LUNDBERG ■ WOESSNER ■ KLUTH

United States Patent Application

COMBINED DECLARATION AND POWER OF ATTORNEY

As a below named inventor I hereby declare that: my residence, post office address and citizenship are as ıted below next to my name; that

I verily believe I am the original, first and joint inventor of the subject matter which is claimed and for which ɔatent is sought on the invention entitled: **TREATMENT OF CARDIOVASCULAR CONDITIONS WITH TRITE**.

ıe specification of which is attached hereto.

I hereby state that I have reviewed and understand the contents of the above-identified specification, ɔluding the claims, as amended by any amendment referred to above.

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I hereby claim foreign priority benefits under 35 U.S.C. §119(a)-(d) or 365(b) of any foreign application(s) r patent or inventor's certificate, or 365(a) of any PCT international application which designated at least one untry other than the United States of America, listed below and have also identified below any foreign application r patent or inventor's certificate having a filing date before that of the application on the basis of which priority is ümed:

› such claim for priority is being made at this time.

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› such claim for priority is being made at this time.

I hereby appoint the following attorney(s) and/or patent agent(s) to prosecute this application and to transact business in the Patent and Trademark Office connected herewith:

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ack, David W.	Reg. No. 42,331	Kim, John P.	Reg. No. 38,514	Peterson, David C.	Reg. No. 47,857
ennan, Thomas P.	Reg. No. 35,075	Klima-Silberg, Catherine L	Reg. No. 40,052	Prout, William F.	Reg. No. 33,995
adwick, Robin A.	Reg. No. 36,477	Kluth, Daniel J.	Reg. No. 32,146	Rodriguez, Richard U.	Reg. No. 45,980
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ochran, David R.	Reg. No. 46,632	Lundberg, Steven W.	Reg. No. 30,568	Schwegman, Micheal L.	Reg. No. 25,816
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ake, Eduardo E.	Reg. No. 40,594	Mates, Robert E.	Reg. No. 35,271	Spiegel, Jack	Reg. No. 34,477
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rguson, Steven	Reg. No. 38,488	Mehrle, Joseph P.	Reg. No. 45,535	Stordal, Leif T.	Reg. No. 46,251
iley, Stephen	Reg. No. 36,357	Muller, Mark V.	Reg. No. 37,509	Tong, Viet V.	Reg. No. 45,416
rrest, Bradley A.	Reg. No. 30,837	Nama, Prakash	Reg. No. 44,255	Viksnins, Ann S.	Reg. No. 37,748
rrie, Gregory J.	Reg. No. 36,530	Nelson, A. James	Reg. No. 28,650	Woessner, Warren D.	Reg. No. 30,440
eaves, John N.	Reg. No. 40,362	Nielsen, Walter W.	Reg. No. 25,539	Marlene Astor	Reg. No. 46,005

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Telephone No. (612)373-6900

I hereby declare that all statements made herein of my own knowledge are true and that all statements made on information and lief are believed to be true; and further that these statements were made with the knowledge that willful false statements and the like so ade are punishable by fine or imprisonment, or both, under Section 1001 of Title 18 of the United States Code and that such willful false tements may jeopardize the validity of the application or any patent issued thereon.

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Alan N. Schechter

1.56 Duty to disclose information material to patentability.

(a) A patent by its very nature is affected with a public interest. The public interest is best served, and the most effective patent amination occurs when, at the time an application is being examined, the Office is aware of and evaluates the teachings of all information terial to patentability. Each individual associated with the filing and prosecution of a patent application has a duty of candor and good th in dealing with the Office, which includes a duty to disclose to the Office all information known to that individual to be material to tentability as defined in this section. The duty to disclose information exists with respect to each pending claim until the claim is canceled withdrawn from consideration, or the application becomes abandoned. Information material to the patentability of a claim that is ıceled or withdrawn from consideration need not be submitted if the information is not material to the patentability of any naining under consideration in the application. There is no duty to submit information which is not material to the patentability of any isting claim. The duty to disclose all information known to be material to patentability is deemed to be satisfied if all information known be material to patentability of any claim issued in a patent was cited by the Office or submitted to the Office in the manner prescribed by 1.97(b)-(d) and 1.98. However, no patent will be granted on an application in connection with which fraud on the Office was practiced attempted or the duty of disclosure was violated through bad faith or intentional misconduct. The Office encourages applicants to efully examine:

(1) prior art cited in search reports of a foreign patent office in a counterpart application, and

(2) the closest information over which individuals associated with the filing or prosecution of a patent application believe any pending claim patentably defines, to make sure that any material information contained therein is disclosed to the Office.

(b) Under this section, information is material to patentability when it is not cumulative to information already of record or being ıde of record in the application, and

(1) It establishes, by itself or in combination with other information, a prima facie case of unpatentability of a claim; or

(2) It refutes, or is inconsistent with, a position the applicant takes in:

(i) Opposing an argument of unpatentability relied on by the Office, or

(ii) Asserting an argument of patentability.

prima facie case of unpatentability is established when the information compels a conclusion that a claim is unpatentable under the ponderance of evidence, burden-of-proof standard, giving each term in the claim its broadest reasonable construction consistent with the cification, and before any consideration is given to evidence which may be submitted in an attempt to establish a contrary conclusion of entability.

(c) Individuals associated with the filing or prosecution of a patent application within the meaning of this section are:

(1) Each inventor named in the application:

(2) Each attorney or agent who prepares or prosecutes the application; and

(3) Every other person who is substantively involved in the preparation or prosecution of the application and who is associated with the inventor, with the assignee or with anyone to whom there is an obligation to assign the application.

(d) Individuals other than the attorney, agent or inventor may comply with this section by disclosing information to the attorney, nt, or inventor.